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13. ABSTRACT *(Maximum 200 Words)*
Approximately 40% of breast cancer patients have tumors containing alterations in the p53 tumor suppressor gene; these patients are known to have a poorer prognosis than those lacking p53 mutations. Arg-His (R-H) mutations are frequently observed at amino acid 175 of p53 (equivalent to murine 172) in such tumors. Expression of a mammary-targeted p53 172R-H transgene rarely induces spontaneous tumors in mice, but carcinogen-treated p53 172R-H transgenic mice develop tumors much earlier than controls. Furthermore, expression of this transgene predisposes tumors induced by carcinogen treatment or oncogene coexpression to the development of aneuploidy. The objective of this proposal is to examine the mechanism by which this particular p53 mutant promotes aneuploidy and tumorigenesis. This will be accomplished through both analysis of gene transcription and analysis of the mitotic machinery and cellular apoptosis. Recent *in vitro* studies indicate that expression of the p53 175R-H mutant in mammary epithelial cells may promote mammary tumorigenesis by deregulating centrosome replication and reducing both basal and DNA-damage-induced apoptosis. Transcriptional regulation studies are currently in progress to identify possible novel protein-protein interactions between the mutant and other proteins leading to the up- or down-regulation of genes linked to the promotion of genomic instability and cancer.

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Introduction

Approximately 50% of human cancers have p53 alterations, and patients with these cancers have poorer prognoses. When functional, the p53 protein blocks proliferation of cells that have sustained DNA damage and induces apoptosis in cells too badly damaged to undergo repair. Mutant forms of p53 are often no longer protective, and in many cases have acquired additional functions which make them more deleterious than the simple absence of wild-type p53. The p53 175R-H mutant is one such gain-of-function mutant. It is a confomational mutant and can no longer bind DNA, although it retains its ability to bind other proteins. It is hypothesized that mutant p53 proteins may gain novel functions by interacting with other proteins that supply a DNA-binding domain. The resulting complex could then use the p53 transactivation domain to modulate a novel set of genes.

p53 has also been implicated in the regulation of the G2/M spindle checkpoint and mitosis, with particularly striking effects upon centrosome duplication. Centrosomal hypertrophy is implicated in at least two processes that adversely affect prognosis in cancer patients: 1) loss of cell polarity and tissue organization, and 2) an increased occurance of multipolar mitoses, which predisposes to the development of aneuploidy. In addition, centrosomes have been shown to be larger and more numerous in high-grade breast adenocarcinomas. Unraveling the mechanism behind the involvement of the 172R-H mutant in the dysregulation of centrosome duplication leading to aneuploidy is of great interest to this project and could help us to better understand tumor progression.

A p53 172R-H transgenic mouse model was generated in the laboratory, and studies of this mouse indicated that the presence of this p53 mutant did not decrease apoptosis or increase proliferation, but did promote the development of aneuploid tumors following carcinogen treatment. Similar results were seen when these mice were crossed with mice carrying other mammary-targeted oncogenes; tumors arising in the p53 mutant-carrying bitransgenic mice were frequently aneuploid. The current studies were initiated in order to address the mechanistic issues: specifically, how the p53 175R-H mutant contributes to mammary tumorigenesis and the development of genomic instability.

Body

Specific Aims:

In order to better understand the role of the p53 172R-H mutant in mammary tumorigenesis, we have modified or extended the scope of the original specific aims of the grant proposal to address new issues raised by experimental results.

1) To study the effect of the WAP-p53 172R-H transgene on genomic instability in early stages of mammary tumorigenesis

2) To investigate p53 172R-H as a gain-of-function mutant by identification of potential indirect target genes and/or novel protein-protein interactions

Progress toward these aims:

Specific Aim 1: To study the effect of the WAP-p53 172R-H transgene on genomic instability in early stages of mammary tumorigenesis.

Initial characterization of mammary tumors arising spontaneously (following pituitary isograft) in mice bitransgenic for WAP-p53 172R-H and WAP-TGF-_ indicated that 57% of tumors arising in the mutant p53-expressing bitransgenic mice were aneuploid, whereas no aneuploid tumors were observed in the WAP-TGF-__control mice. Both sets of tumors arise with a latency of 100-110 days post-isograft. Loss of wild-type p53 is known to influence aneuploidy through dysregulation of centrosome duplication. Furthermore, centrosomes from high-grade human breast cancers are abnormal in many respects, indicating that there may be a relationship between centrosome abnormality and aneuploidy, which is a marker of poor prognosis. It has been shown that aberrant centrosome duplication can be detected in early hyperplasias and even in phenotypically normal, but transformed, tissue. We initially hypothesized that the aneuploidy we had observed in bitransgenic tumors might be accompanied by centrosome dysregulation. Furthermore, given the short timeframe in which both of these sets of tumors arose, we could examine mammary glands of mice at defined timepoints within the 110 day window for aberrant centrosome numbers occurring in phenotypically normal tissue and preceding the development of aneuploid tumors.

Although preliminary results indicated that there might be more centrosomes in bitransgenic tumors, the complete study did not bear this out. Neither group of tumors appeared to contain aberrant centrosome numbers, despite the ploidy variance. Furthermore, the pretumor samples from both groups appeared to have normal numbers of centrosomes at the timepoints examined, which were 15, 30, 45, 60, and 90 days post-isograft.

However, this did not discount the possiblity that some transient centrosome abnormality resulting in later ploidy-related consequences might be induced very soon (*i.e.*, less than 15 days) after expression of the p53 172R-H mutant in the mouse mammary gland. In order to address this possibility, we extended this specific aim to include *in vitro* studies. p53-null mammary epithelial cell [MEC] cultures were transfected with either wild-type p53 or p53 172R-H using an adenoviral method, and subsequently immunostained for both p53 and gamma-tubulin, a component of centrosomes. Transfected (*i.e.*, p53-positive) cell centrosome numbers were analyzed and compared to those of untransfected cells. These experiments indicated that cells transfected with the p53 mutant did indeed demonstrate aberrant centrosome numbers by 3 days post-transfection, whereas this was not seen in mock-transfected cells or cells transfected with wild-type p53. However, very few MECs stably-transfected with the mutant p53 had aberrant centrosome numbers, suggesting that the majority of the abnormal cells seen in the transient transfection experiments underwent apoptosis due to failure in mitosis. Additional experiments indicated that MECs stably transfected with the p53 mutant displayed reduced apoptosis, both basal and DNA-damage induced, suggesting a second mechanism by which this p53 mutant might also contribute to mammary tumorigenesis. We hypothesize that the early centrosome abnormalities create a cellular environment in which genomic instability is more frequent than usual, compounded by diminished apoptotic capabilities in the same cells, and that this forms the basis of the tumor predisposition seen in the mouse models.

Specific Aim 2: p53 172R-H as a gain-of-function mutant: the identification of potential indirect target genes and/or novel protein-protein interactions

It is known that the p53 172R-H mutant cannot bind to p53 consensus binding sites, but there are several papers suggesting that p53 may have indirect transcriptional effects. Furthermore, it is possible that this p53 mutant may mediate its gain-of-function effects partially through aberrant protein-protein interactions. Any genes up- or down-regulated preferentially in the presence of the mutant could be directly involved in the dysregulation of normal centrosome number and maintenance of chromosomal stability.

The potential transcriptional effects of the p53 172R-H mutant are currently being addressed. Initially, we proposed to use cDNA obtained from p53-null MECs transiently transfected with mutant p53, wild-type p53, or mock-transfected cells to screen Atlas™ Arrays (Clontech) for differentially expressed genes. However, due to difficulties with data analysis, we were not confident regarding the validity of data obtained from these screens, and so took an alternative approach.

We employed CLONTECH's PCR-Select™ cDNA Subtraction Kit to perform a suppressive subtractive hybridization in which cDNA made from p53 null cells transfected with wildtype p53 was subtracted from cDNA made from mutant p53 transfected cells. The subtraction procedure generated a pool of cDNAs differentially expressed in the presence of the mutant protein two days after transfection. These cDNAs were then cloned into pGEM vectors; 900 plasmids were differentially screened with forward and reverse subtracted probes, and 120 clones were chosen to be sequenced following the screening. Of the102 clones identified in sequence homology databases, many interesting genes were revealed to be candidates for transcriptional regulation by mutant p53. A wide variety of genes were identified – some of these include developmental (EED), metabolic, transcriptional (TRAP100, SRA), translational (Trt, Naca), and structural genes (γ-actin), as well as genes involved in transformation (MAT1), signal transduction (SDF1, $G\alpha_s$, Pitpn, MRK, calcyclin), chromatin remodeling (Psma1, Hmg1, H2A), DNA repair (Ku70) and apoptosis (Naip1). In addition, 11 ESTs were identified and are currently being investigated. We are especially interested in the genes involved in transcription, chromatin remodeling, and DNA repair and plan to pursue the involvement of mutant p53 in these processes in an attempt to discover its role in mammary carcinogenesis. Following the verification of up- or down-regulation of these genes by p53 172 R-H, the interaction between mutant p53 and the known transcriptional regulators of these genes will be investigated. It is expected that novel protein-protein interactions between the mutant and other proteins directly or indirectly involved in transcription of key genes involved in tumor progression will be revealed.

Key Research Accomplishments

- Determined that centrosome amplification is not necessarily coincident with aneuploidy in a bitransgenic mouse mammary tumor model
- Optimized adenoviral transduction of p53-null MECs
- Demonstrated transient centrosome abnormalities in p53 175R-H-transfected MECs
- Demonstrated that stably-transfected cell populations mimic tumor populations in that they lack significant percentages of cells with centrosome abnormalities
- Demonstrated that MECs stably transfected with p53 175R-H display diminished basal and DNA damage-induced apoptotic responses
- Generated a subtractive cDNA library containing cDNAs differentially expressed in p53 null MECs transiently transfected with p53 172 R-H
- Revealed important and significant candidate genes possibly regulated by mutant p53, such as Hmg1, Ku70, SRA, and MAT1

Reportable Outcomes

Data from this project was presented at two national meetings:

1. Molecular Biology and Pathology of Neoplasia Workshop, Keystone, CO, July 1998.
2. Mouse Models of Mammary Tumorigenesis Meeting, Bar Harbor, ME, October, 1999.

Data from this project has been published in two peer-reviewed journal articles:

1. Murphy, K.L., and Rôsen, J.M. (2000) Mutant p53 and genomic instability in a transgenic mouse model of breast cancer. *Oncogene*, **19**:1045.

2. Murphy, K.L., Dennis, A.P, and Rosen, J.M. (2000) A gain-of-function p53 mutant promotes both genomic instability and cell survival in a novel p53-null mammary epithelial cell model. *FASEB J.*, in press.

Conclusions

Discovering the role played by mutant p53 in the development of breast cancer is the primary goal of this study. We believe that the p53 172 R-H mutant contribution to cancer progression is primarily due to different protein-protein interactions conferred by the mutant with proteins that would otherwise interact differently, or not at all, with wildtype p53. These new or aberrant interactions could then disrupt the cells' ability to successfully maintain the integrity of the genome either directly by disrupting proper mitotic processes (including centrosome duplication) or indirectly, by altering gene expression via another protein's DNA-binding domain. We are anticipating the identification and further characterization of some of these interactions within the next year.

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Mutant p53 and genomic instability in a transgenic mouse model of breast cancer

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Introduction

Tumorigenesis is a multistage process involving multiple genetic aberrations (Fearon and Vogelstein, 1990). These aberrations may include mutations in cellular proto-oncogenes that constitutively activate a growth signal transduction pathway, inactivation of tumor suppressor genes, and inactivation of genes that promote cell death. Other mutations provide an indirect growth advantage by compromising the genetic stability of the cell, increasing the occurrence of subsequent genetic lesions (Tlsty *et al.*, 1995). Each successive mutation may then enhance the tumorigenic potential of the cell (Nowell, 1976). Advances in transgenic and knockout technologies make it possible to genetically engineer mice to mimic the individual steps in this process in order to better understand events contributing to cancer progression at the molecular level. Specifically, mouse models prone to genetic instability have been generated that may be useful to screen for early molecular events involved in carcinogenesis.

p53 is the most commonly mutated gene in human cancers, with approximately 40% of tumors displaying some alteration in p53 (Osborne *et al.*, 1991). Unlike deletion or nonsense mutations observed in other tumor-suppressor genes, most p53 alterations are missense mutations resulting in the expression of a functionally altered protein (Hainaut *et al.*, 1997). Wild-type p53 has been called the 'guardian of the genome,' as p53 responds to DNA damage or checkpoint failure by either arresting the cell in the G1 phase of the cell cycle for damage repair or through the initiation of an apoptotic pathway to eliminate the damaged cell entirely (Lane, 1992). Wild-type p53 is particularly critical for the maintenance of genomic stability; aberrant ploidy, gene amplification, increased recombination, and centrosomal dysregulation have been observed in cells lacking p53 (Donehower, 1997). Mutations in p53 may result not only in a loss of wild-type function, but also in the generation of dominant-negative and gain-of-function mutants (e.g., Dittmer *et al.* (1993)).

Specific p53 mutations, including those at codon 175, have been associated with a poor prognosis in breast cancer patients, and also with primary resistance to chemotherapy (Aas *et al.*, 1996). This is one of five 'hotspot' codons present in the sequence-specific DNA binding domain of p53 that represent ~20% of all p53 mutations reported (Hainaut *et al.*, 1997). Class II mutations, such as those at codon 175, affect residues crucial for maintenance of the correct orientation of the DNA-binding surface of non-contiguous loops and helices (Cho *et al.*, 1994). Amino acid 175 is not located within the regions of the p53 protein that directly contact DNA, as are most of the other commonly mutated residues. The arginine side chain participates in bonds bridging loops 2 and 3 of the protein (Cho *et al.*, 1994), and several lines of evidence suggest that the protein is at least partially unfolded as a result of the side chain substitution (Cho *et al.*, 1994). The 175 R-H mutant human p53 protein is incapable of binding a consensus p53 DNA-binding site (Kern *et al.*, 1991, 1992; Ory *et al.*, 1994). The unique properties of certain p53 mutants may reflect their selective activation of specific DNA targets (Dittmer *et al.*, 1993; Thukral *et al.*, 1995) and/or participation in novel protein-protein interactions (Chen *et al.*, 1994).

The development of both p53 knockout and p53 mutant transgenic mice has greatly facilitated studies of the role of p53 in carcinogenesis and tumor progression (Donehower, 1996). By crossing p53 null mice with lines of transgenic mice overexpressing specific oncogenes it has been possible to gain new insights into the mechanisms by which different signal transduction pathways interact with p53 to affect tumorigenesis (e.g., Donehower *et al.* (1995)). However, p53 knockout mice have some limitations for experiments designed to determine the role of p53 in mammary tumorigenesis, as these mice frequently die from lymphomas and sarcomas prior to mammary tumor development (Donehower *et al.*, 1992). Consequently, mice containing a mutant p53 transgene targeted specifically to the mammary gland have been generated for these studies. The 175 R-H mutation is the second most-frequent p53 mutation observed in breast cancer, accounting for approximately 6% of those reported to date (Hainaut *et al.*, 1997). In order to study the role of the murine-equivalent 172 R-H mutant p53 protein in mammary tumorigenesis, a genomic minigene construct containing this mutation (Li *et al.*, 1998) was targeted specifically to the mammary gland of transgenic mice using the whey acidic protein (WAP) promoter (Bayna and Rosen, 1990).

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The WAP-p53 172 R-H transgenic model

The 172 R-H transgenic mice exhibit a negligible level of spontaneous tumorigenesis (Li *et al.*, 1997, 1998). Transgene expression alone does not alter normal mammary development at the gross histological level, and as assessed through the analysis of apoptosis during involution and proliferation during pregnancy (Li *et al.*, 1998). The mutant protein also does not alter the expression levels of p21, MDM2, proliferative cell nuclear antigen (PCNA), or several other genes known to be regulated directly by wild-type p53 at the transcriptional level (Li *et al.*, 1998). To determine whether the presence of the transgene predisposed these mice to mammary tumorigenesis, they were given pituitary isografts to stimulate transgene expression (Medina, 1974) and treated with the carcinogen, dimethylbenz(a)anthracene (DMBA). Tumors arising in carcinogen-treated nontransgenic (FVB) and transgenic mice were analysed to determine the mechanisms by which this mutant p53 might promote tumorigenesis in the mammary gland.

Carcinogen susceptibility and tumor analysis

The 172 R-H transgenic mice developed tumors significantly more rapidly than controls, and exhibited a greater tumor burden (Li *et al.*, 1998). One hundred per cent of transgenic mice developed tumors by week 28 post-DMBA treatment, while only 85% of FVB nontransgenic mice developed tumors by week 45 (Li *et al.*, 1998).

Apoptosis and cell proliferation in tumors from transgenic and nontransgenic mice were compared, but no significant differences were found (Li *et al.*, 1998). Loss of p53 function has been shown in the choroid plexus to influence tumorigenesis primarily through the inhibition of apoptosis (Symonds *et al.*, 1994), but this does not appear to be the case in the mammary gland (Jones *et al.*, 1997). However, tumor cell nuclei from the transgenic mice (Figure 1a) were in most cases larger and more pleomorphic than those from tumors arising in nontransgenic mice. Since p53 loss (Cross *et al.*, 1995; Fukasawa *et al.*, 1996) or mutation (e.g., Liu *et al.* (1996)) has been shown to result in genomic instability, the DNA content of populations of tumor cells from transgenic and nontransgenic mice was assessed by flow cytometry. Aberrant ploidy was more often seen in carcinogen-induced tumors from transgenic animals (Figure 1b) than in carcinogen-induced tumors from control animals (Li *et al.*, 1998).

Bitransgenic model systems

Mice carrying the 172 R-H transgene have also been crossed in separate experiments with mice overexpressing MMTV-neu (*erb-B2*) (Li *et al.*, 1997), WAP-*des*-IGF-1 (Hadsell *et al.*, 1999), or WAP-TGF-α (K Murphy and J Rosen, 1999, unpublished results). Co-expression of MMTV-*neu* or WAP-*des*-IGF-1 with WAP-p53 172 R-H significantly decreased tumor latency relative to that seen with the oncogene alone (Hadsell *et al.*, 1999; Li *et al.*, 1997). In the WAP-TGF-α cross, both the bitransgenic and single transgenic TGF-α mice developed tumors with a mean latency of approximately 100 days (Figure 2), and because of this short latency no significant difference between the two groups was observed. Mammary tumors from mice expressing the p53 172 R-H transgene in conjunction with any of these growth factors or receptors were frequently aneuploid (Figure 1f), as assessed by flow cytometry, but tumors from mice expressing only the *neu*, *des*-IGF-1, or TGF-α transgene were not (Figure 1d). Tumors from bitransgenic mice also contained large irregular nuclei (Figure 1e) similar to those seen in tumors from the DMBA-treated transgenic mice (Hadsell *et al.*, 2000, submitted; Li *et al.*, 1997; and K Murphy and J Rosen, 1999, unpublished results).

These results suggest that the p53 172 R-H transgene predisposes female mice to the development of aneuploid mammary tumors once some other initiating event (i.e., oncogene co-expression in the mammary gland or carcinogen treatment) has taken place. Since the expression of the p53 172 R-H transgene alone resulted in very few spontaneous tumors in mice less than a year old (Hadsell *et al.*, 2000, submitted; Li *et al.*, 1997, 1998), while accelerating tumorigenesis caused by both carcinogen treatment and oncogene expression, this appears to be an excellent model system in which to study early events in mammary tumorigenesis. Furthermore, although most advanced-stage human breast cancers are aneuploid, mammary tumors generated in most mouse model systems to date have been uniformly diploid, which limits the utility of these models. This is one of the few model systems that consistently generates aneuploid tumors similar to grade 3, high S phase, hormone-independent human breast cancers. Patients with these types of tumors usually have the poorest prognosis. Other model systems generating aneuploid mammary tumors include p53-deficient mammary gland (Jerry, this volume), p53-deficient *Wnt-1* transgenic mice, which develop tumors exhibiting recurring changes on several chromosomes (Donehower *et al.*, 1995), MMTV-*neu* mice, which frequently develop mammary tumors exhibiting loss of heterozygosity on chromosome 4 (Ritland *et al.*, 1997), C3-driven SV40 Tag transgenic mice, which develop mammary tumors consistently showing DNA gains on chromosome 6, and WAP-Str1 (stromelysin-1) mice, which develop mammary lesions containing consistent genomic changes (Sternlicht *et al.*, 1999).

Interestingly, although the WAP-TGF-α transgene is a potent mammary oncogene (expression results in short mammary tumor latency), when these mice are crossed with mice carrying another codon 172 p53 mutant generated in our laboratory, WAP-p53 172 R-L (Figure 2), mammary tumorigenesis is almost completely prevented (K Murphy and J Rosen, 1999, unpublished observations). When the p53 172 R-L mice and control nontransgenics were treated with the carcinogen DMBA, mammary tumorigenesis was delayed in the transgenic mice because of high levels of mammary epithelial cell apoptosis ($\sim$20%) induced by this transgene, which retains many properties of wild-type p53 (Li *et al.*, 1995). Presumably the p53 172 R-L transgene is blocking tumorigenesis in the WAP-p53 172 R-L/WAP-TGF-α bitransgenic mice by a similar mechanism. The difference in tumorigenic properties between the 172 R-H and 172 R-L p53 mutants is striking, given that they occur at the same codon and are both found in human breast tumors.

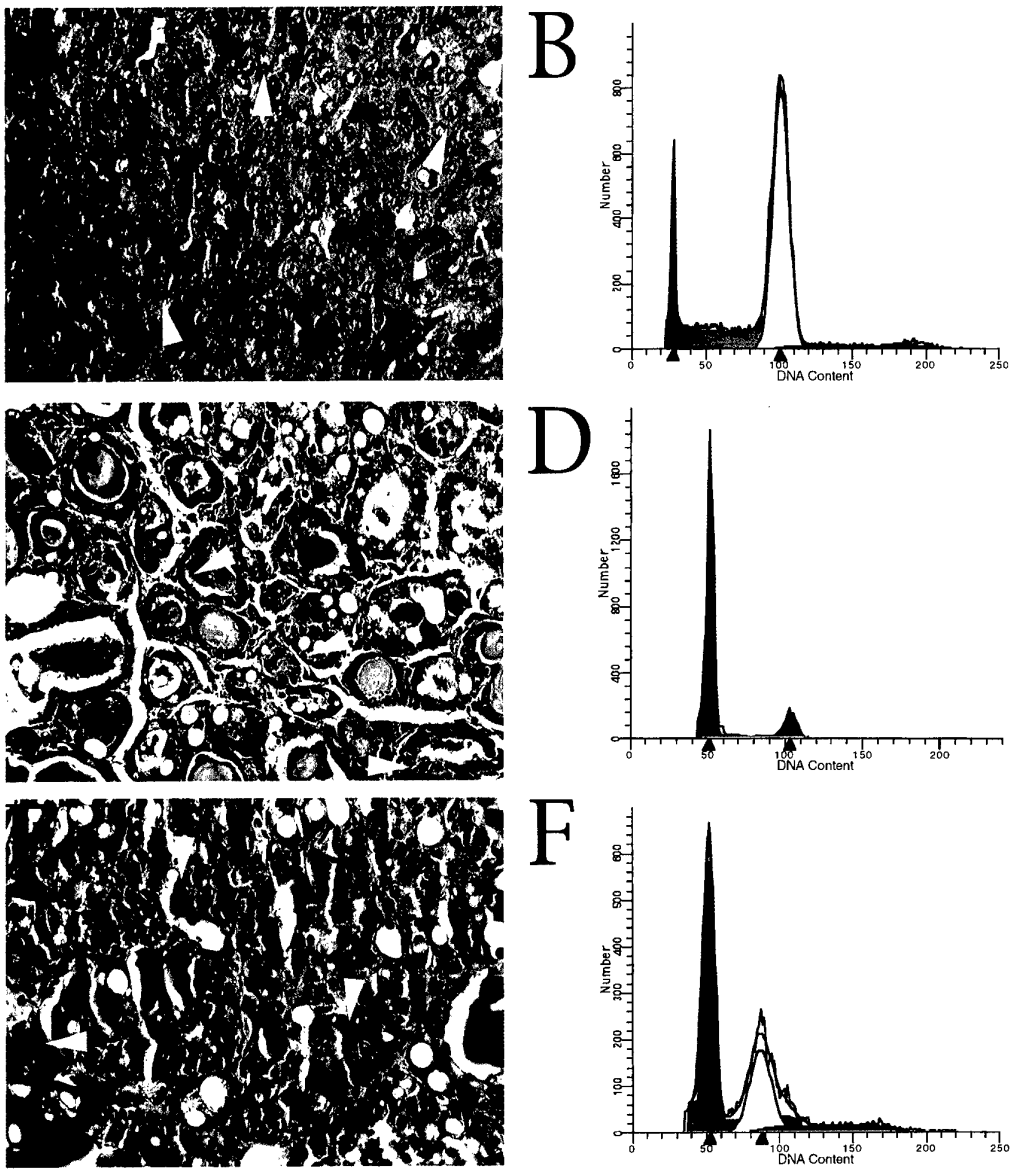

Figure 1 Histology and ploidy analysis of transgenic mouse tumors. (**a**), (**c**) and (**e**) Hematoxylin-and-eosin stained sections of tumors arising in representative WAP-p53 172 R-H (spontaneous), WAP-TGF-α, and WAP p53 172 R-H/WAP-TGF-α bitransgenic mice respectively, while (**b**), (**d**) and (**f**) represent flow cytometric analyses of the same tumors. The white arrowheads in (**a**) and (**e**) indicate representative large irregular nuclei, while the arrowheads in (**c**) point to normal-sized nuclei for comparison. The yellow peaks in (**b**) and (**f**) indicate the presence of aneuploid populations of cells in the WAP-p53 172 R-H and WAP-p53 172 R-H/WAP-TGF-α tumors

p53 172 R-H as a gain-of-function mutant

The 172 R-H p53 protein is a dominant-negative mutant in that it can interact with wild-type p53, but is no longer capable of specific DNA binding (Kern *et al.*, 1991, 1992; Ory *et al.*, 1994). This mutant, therefore, loses many of the direct transcriptional regulatory capabilities of wild-type p53. However, it appears to confer novel functions, indicating that it is a gain-of-function mutant. For example, it is capable of stimulating expression of MDR-CAT (a human multi-drug resistance {MDR}-1 gene promoter-CAT construct) in p53-null cells, in a manner reversible by co-transfection of wild-type p53 (Chin *et al.*, 1992). When the 175 R-H mutant was transfected into p53-null Saos-2 cells, it conferred a growth advantage. Injection of a cell line expressing this mutant p53 protein into nude mice resulted in tumorigenesis, which was not seen with the parental p53-null cells (Dittmer *et al.*, 1993). This mutant protein was also able to cooperate in co-transfection experiments with activated H-*ras* in the transformation of rat embryo fibroblasts (Hinds *et al.*, 1990). Furthermore, cells containing this p53 mutation exhibit a dominant gain-of-function defect in spindle (G2/M) checkpoint control. When incubated with colcemid, a spindle assembly inhibitor, cells containing wild-type p53 arrest with 4n DNA content, but cells containing this p53 mutant can re-enter S phase and subsequently become polyploid (Gualberto *et al.*, 1998). p53 has been implicated in the regulation of the G2/M spindle checkpoint and mitosis (Cross *et al.*, 1995; Fukasawa *et al.*, 1996;

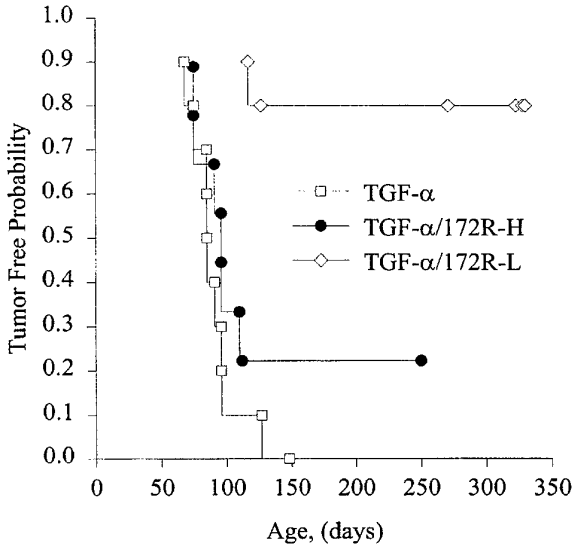

Figure 2 Tumor latency in pituitary isografted WAP-TGF-α, WAP-TGF-α/WAP-p53 172 R-H, and WAP-TGF-α/WAP-172 R-L female mice. The rapid (~100 days) mammary tumorigenesis induced by the WAP-TGF-α transgene is not accelerated by the WAP-p53 172 R-H transgene in the WAP-TGF-α/WAP-p53 172 R-H bitransgenic females. However, the WAP-p53 172 R-L transgene almost completely eliminates mammary tumorigenesis in the WAP-TGF-α/WAP-p53 172 R-L bitransgenic females, indicating that the latter p53 transgene has a protective effect

Gualberto *et al.*, 1998; Paulovich *et al.*, 1997; Stewart *et al.*, 1995), with particularly striking effects upon centrosomal duplication (Fukasawa *et al.*, 1996).

Primary skin tumors from mice bearing a skin-targeted p53 172 R-H mutation (murine amino acid 172 is equivalent to human 175) display a much greater degree of aberrant centrosomal duplication than do tumors from p53-null mice (Wang *et al.*, 1998). Centrosomal amplification is implicated in at least two processes that adversely affect prognosis in cancer patients: (1) loss of cell polarity and tissue organization, and (2) an increased occurrence of multipolar mitoses, which predisposes to the development of aneuploidy, as it promotes unequal division of genetic material (Lingle *et al.*, 1998). A recent study found that centrosomes in high-grade breast adenocarcinoma cells are larger and more numerous and contain more centrioles and pericentriolar material than do normal breast specimens. They are also inappropriately phosphorylated, and nucleate abnormally large numbers of microtubules (Lingle *et al.*, 1998).

Approximately 50% of mammary tumors arising in the WAP-172 R-H/WAP-TGF-α bitransgenics in our experiments were aneuploid, while aberrant ploidy was not seen in any of the tumors arising in similarly treated WAP-TGF-α single transgenic females. As both p53 loss and p53 mutation have been associated with centrosome dysregulation and aneuploidy, centrosome numbers in thick frozen sections from both groups of mammary tumors were assessed by confocal microscopy using standard (fluorescent) immunohistochemical techniques. Surprisingly, neither group of tumors demonstrated centrosome abnormalities above background levels (with 'background' defined as $\leqslant 10\%$ by BR Brinkley, personal communication), despite the differences in tumor ploidy (K Murphy, BS Kolle, T Goepfert, J Zhong, BR Brinkley and J Rosen, 1999, unpublished observations).

In order to eliminate the possibility that centrosome dysregulation was occurring in the bitransgenic females in early stages of mammary tumorigenesis (i.e., before frank tumors were discovered) and promoting the later development of aneuploid tumors in that manner, a study of precancerous mammary glands was performed in this system. As discussed above, both the bitransgenic WAP-p53 172 R-H/WAP-TGF-α and the single-transgenic WAP-TGF-α females develop mammary tumors within approximately 100 days following the surgical implantation of a pituitary isograft to stimulate transgene expression, providing a reasonably short window in which to look for early centrosomal dysregulation induced by the p53 172 R-H transgene. Isografts were given to additional groups of young bitransgenic and WAP-TGF-α females, and also to single transgenic WAP-p53 172 R-H and nontransgenic (FVB) females for comparison. Mammary glands were surgically excised from these groups of mice at defined timepoints following isografting (15d, 30d, 45d, 60d, and 90d) and analysed as above for centrosome amplification. The histology of some of these lesions can be found at http://mammary.nih.gov/cgi-bin/imaged_b/output.taf. Again, no abnormal centrosome numbers were observed in any of the four groups of mice, at any timepoint (K Murphy and J Rosen, 1999, unpublished observations). These studies suggest that while centrosome dysregulation is known to promote genomic instability and tumorigenesis, it is not a prerequisite for the development of aneuploid tumors in the mouse mammary gland. However, we cannot formally exclude the possibility that aberrant centrosome duplication leading to genetic instability in a subpopulation of cells may be occurring before the 15-day timepoint in our studies. Most cells with an aberrant number of centrosomes would be predicted to undergo apoptosis following multipolar cell division, but a few may survive, giving rise to a potentially pretumorigenic subpopulation of cells that might not have been detected in these assays.

Currently, there is much debate as to the precise relationship between aberrant centrosome duplication and aneuploidy. Our results with the p53 172 R-H transgenic mice support the hypothesis that centrosome amplification does not necessarily precede the development of aneuploidy. This is further supported by a study of aneuploid mouse mammary tumors that arise in hormonally-treated Balb/c mice that have had syngeneic p53-null mammary epithelial cells transplanted into their cleared fat pads, which showed that very few of these tumors had aberrant numbers of centrosomes (D Medina, personal communication). Conversely, in a NMU-induced rat mammary tumor system, aberrant centrosome numbers are seen, but the tumors are near-diploid (BR Brinkley, personal communication). In contrast, when MCF7 (BR Brinkley, personal communication) and MCF10A (Zhou *et al.*, 1998) cells are transfected with the centrosome-associated kinase BTAK, centrosome amplification and aneuploidy are seen concurrently. At present, it appears that centrosome amplification may neither be necessary nor sufficient to induce aneuploidy, but that it does constitute one mechanism by which aneuploidy may be initiated.

Recently it has been suggested that at least some genomic instability is the result of failures in the DNA repair pathway. Wild-type p53 has been reported to interact with BRCA1 (Chai *et al.*, 1999; Zhang *et al.*, 1998), BRCA2, and RAD51 (Marmorstein *et al.*, 1998; Sharan *et al.*, 1997). Thus, this multiprotein complex (Figure 3) may play an important role in DNA repair (Chen *et al.*, 1999; Patel *et al.*, 1998; Zhang *et al.*, 1998). BRCA1 is also important for the cellular responses to DNA damage that are mediated through the hRad50-hMre11-p95 complex (Zhong *et al.*, 1999). BRCA1 is known to physically associate with components of the RNA polymerase II general transcriptional apparatus, suggesting a role in transcriptional control and DNA repair (Chen *et al.*, 1999) especially as BRCA1 is accompanied by Rad51 when it relocates to PCNA-positive replication sites following hydroxyurea or low-dose UV treatment of cells (Chen *et al.*, 1999). Rad51 mutants fail to correctly repair double-stranded DNA breaks (Shinohara *et al.*, 1992). BRCA1 is also required for transcription-coupled repair of oxidative DNA damage (Gowen *et al.*, 1999). Fibroblasts derived from embryos carrying a targeted exon 11 BRCA1 deletion have a defective G2/M checkpoint, which is accompanied by extensive chromosomal abnormalities. They also contain multiple functional centrosomes, leading to unequal chromosome segregation and aneuploidy (Xu *et al.*, 1999). BRCA1 is known to associate with the centrosome during mitosis (Hsu and White, 1998). Tumors carrying mutations in BRCA2 also show complex chromosomal changes (Gretarsdottir *et al.*, 1998; Patel *et al.*, 1998).

Wild-type p53 may itself play a direct role in DNA repair. It is known to preferentially bind free DNA ends, single-stranded DNA, short mismatched loops and radiation damaged DNA, and it can reanneal DNA strands (Donehower, 1997). It can also bind DNA-repair associated proteins such as ERCC3, RPA, XPB, and XPD and colocalize with them to sites of DNA repair (Donehower, 1997). It is conceivable that the 172 R-H mutant p53 protein may, therefore, be promoting genomic instability at least partially through disrupting the normal function of DNA repair complexes (Figure 3). It has also been suggested that mitotic checkpoint inactivation (such as that induced by p53 175 R-H [Gualberto *et al.*, 1998]) may cooperate with BRCA2 deficiency to promote tumorigenesis in humans (Lee *et al.*, 1999), lending further support to the hypothesis that disrupted interactions between members of the BRCA1/BRCA2/Rad51/p53 complex may be integrally involved in mammary tumorigenesis.

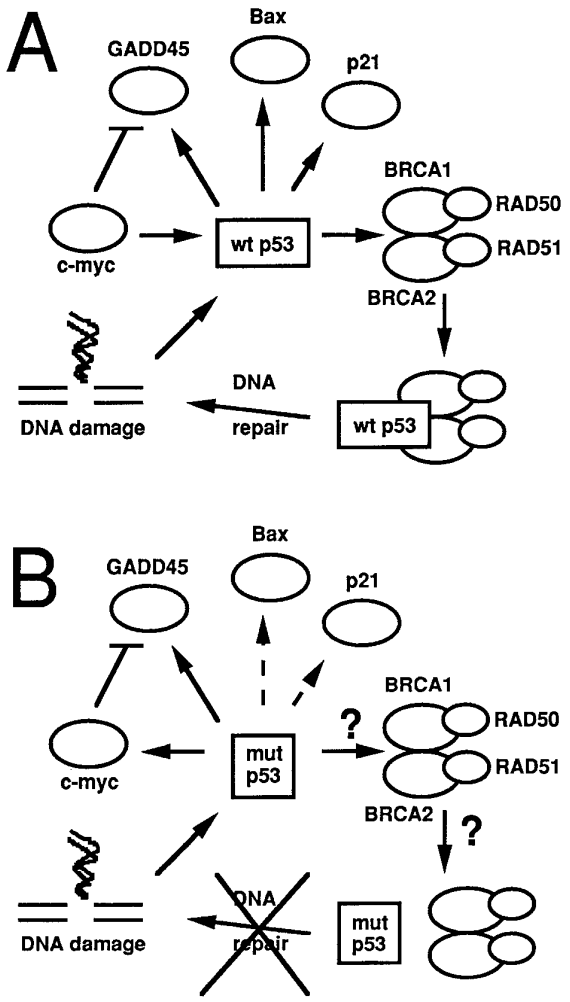

Figure 3 Differential functions of wild-type and mutant p53. (**a**) Depicts transactivational and protein-protein interactions made by wild-type p53, while (**b**) depicts the effect of the p53 172 R-H mutant p53 on the same interactions. Dotted lines in (**b**) indicate transactivational interactions that are no longer functional. We hypothesize that the wild-type p53 protein contributes to DNA repair after damage through its interaction with the BRCA1/BRCA2/Rad51 complex, an interaction that may not be functional when there are mutations in the p53 protein

Potential gain-of-function mechanisms

As p53 is a multifunctional protein, the p53 gain-of-function mutants may lose the ability to regulate transcription of certain target genes involved in cell cycle control and apoptosis, like p21 or Bax, that require DNA binding, but still retain other functions that require protein-protein interactions (Figure 3). The latter may fall into several categories. First, interactions with other transcription factors or co-activators could lead to transcriptional activation from novel promoters. It has recently been reported that p53 participates in transcriptional induction of the *GADD45* gene through an interaction with WT-1 bound to an Egr-1 site on the GADD45 promoter, but not as a result of direct DNA binding by p53 (Zhan *et al.*, 1998). Second, nonsequence-specific interactions of the p53 carboxy-terminus with single-stranded DNA or RNA could affect gene regulation. For example, it has been reported that p53 mutants can induce c-*myc* gene expression through an interaction between the carboxy-terminal region of p53, which possesses a single-stranded DNA and RNA binding activity, and a region located at the exon 1/intron 1 boundary of c-*myc* (Frazier *et al.*, 1998). This interaction may overcome the block to transcriptional elongation known to occur in the c-*myc* gene. Finally, nontranscriptional interactions such as those already known to exist between wild-type p53 and centrosome elements/microtubules (e.g., Brown *et al.* (1994)) could affect mitotic fidelity and genomic stability in early

tumor development. These mechanisms may account for the apparent 'gain-of-function' and predisposition to genomic instability that have been observed not only in transgenic mice overexpressing WAP-172 R-H p53, but also in cell culture systems.

Conclusions

Cancer initiation and progression are complex processes involving many genetic and epigenetic factors. One of the future goals of the National Cancer Institute is the development of improved mouse models to help elucidate the mechanisms underlying these processes and for use in testing new diagnostic and therapeutic regimens. In this regard, the WAP-p53 172 R-H transgenic model developed in our laboratory is unique in that it consistently produces tumors characteristic of high-grade breast adenocarcinomas. This model should, therefore, serve as an excellent system in which to study the mechanisms responsible for genetic instability and may help identify those factors that promote tumor progression and metastasis. Finally, because mammary gland abnormalities are rarely observed in this model in the absence of carcinogen administration or oncogene co-expression, this model should facilitate the identification of earlier genetic lesions.

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A gain of function p53 mutant promotes both genomic instability and cell survival in a novel p53-null mammary epithelial cell model

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ABSTRACT **Approximately 40% of human breast cancers contain alterations in the tumor suppressor p53. The p53 172R-H gain-of-function mutant (equivalent to the common 175R-H human breast cancer mutant) has been shown to promote aneuploidy and tumorigenesis in the mammary gland in transgenic mice and may affect genomic stability in part by causing centrosome abnormalities. The precise mechanism of action of these gain-of-function mutants is not well understood, and has been studied primarily in fibroblast cell lines. A novel p53-null mouse mammary epithelial cell line developed from p53-null mice has been used in adenovirus-mediated transient transfection experiments to study the properties of this p53 mutant. Marked centrosome amplification and an increased frequency of aberrant mitoses were observed within 72 h of introduction of p53 172R-H. However, few cells with aberrant centrosome numbers were observed in cells stably expressing the p53 172R-H mutant. Furthermore, stable expression of this p53 mutant reduced both basal and DNA damage-induced apoptosis. This result may be mediated in part through abrogation of p73 function. The p53 172R-H mutant, therefore, appears to influence tumorigenesis at the molecular level in two distinct ways: promoting the development of aneuploidy in cells while also altering their apoptotic response after DNA damage.—Murphy, K. L., Dennis, A. P., Rosen, J. M. A gain of function p53 mutant promotes both genomic instability and cell survival in a novel p53-null mammary epithelial cell model. *FASEB J.* 14, 000–000 (2000)**

Key Words: mutant p53 · apoptosis · aneuploidy · centrosome abnormalities

WILD-TYPE P53 ACTS as the 'guardian of the genome' (1), responding to DNA damage or checkpoint failure by either arresting cell cycle progression to facilitate DNA repair or initiating an apoptotic pathway to remove damaged cells. Wild-type p53 is critical for the maintenance of genomic stability: aberrant ploidy, gene amplification, increased recombination, and centrosomal dysregulation have all been observed in cells lacking p53 (2). p63 and p73, two other proposed members of the p53 family, may compensate for wild-type p53 under some circumstances (3).

Tumorigenesis is a multistage process involving multiple genetic aberrations (4). p53 is the most commonly mutated gene in human cancers, with ~40% of tumors displaying some genetic alteration (5). Most p53 alterations are missense mutations (6) that may be accompanied by loss of the remaining wild-type allele. The p53 175 Arg-His (R-H) mutation makes up ~5% of all p53 mutations found in breast cancer and is the third most frequent p53 mutation (ref 6; and see web site at http://perso.curie.fr/Thierry.Soussi/p53_databaseWh.htm). Specific p53 mutations, including those at codon 175, have been associated with poor prognosis in breast cancer patients and also with primary resistance to chemotherapy (7).

Mutations in p53 may result in loss of wild-type function or generation of dominant-negative and gain-of-function mutants (e.g., ref 8). The 175R-H p53 protein is a dominant-negative mutant that can interact with wild-type p53, but is no longer capable of specific DNA binding (6, 9–11) and so loses many of the direct transcriptional regulatory capabilities of wild-type p53. It also appears to confer novel functions, indicating that it is a gain-of-function mutant (see, for example, ref 12). However, these studies have been performed in either fibroblasts or non-mammary epithelial cell lines usually containing multiple genetic aberrations in addition to p53 loss or mutation. To better understand the role of mutant p53 in mammary tumorigenesis, it is critical to use the appropriate cell system (mammary epithelial cells) containing a minimal number of other genetic aberrations and no competing wild-type (or other forms of mutant) p53. A novel cell system meeting

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these criteria has been developed and was used for the current experiments.

One of the gain-of-function phenotypes previously reported for the 172R-H mutant relates to centrosome number and ploidy. Primary skin tumors from mice bearing a skin-targeted p53 172R-H mutation (murine amino acid 172 is equivalent to human 175) display a much greater degree of aberrant centrosomal duplication than do tumors from p53-null mice (13). Normal nondividing cells have one centrosome and dividing cells two, which form the poles of the spindle (14). Centrosomal amplification is implicated in two processes adversely affecting prognosis in cancer patients: *1)* loss of cell polarity and tissue organization, and *2)* increased occurrence of multipolar mitoses, which promotes unequal genomic segregation (15). A recent study found that centrosomes in high-grade breast adenocarcinoma cells are larger and more numerous than those from normal breast specimens (14). Centrosome amplification induces chromosomal instability (16), which is associated with tumor aneuploidy (17).

A transgenic mouse model has previously been generated to explore the role of the murine p53 172R-H protein in mammary tumorigenesis (18). A genomic minigene construct containing this mutation was targeted specifically to the murine mammary gland using a whey acidic protein (WAP) promoter (19). Transgene expression resulted in very few spontaneous tumors, but predisposed mice to the development of mammary tumors once some initiating event (e.g., carcinogen treatment or oncogene coexpression in the mammary gland) had taken place (12, 18, 20, 21). These tumors were frequently aneuploid (12, 18, 20, 21).

Studies were initiated using the unique p53-null mammary epithelial cell line in order to dissect the mechanism(s) by which the p53 172R-H mutant promotes aneuploidy and tumorigenesis in the mouse mammary gland. The results of these studies suggest that the p53 172R-H mutant may play a dual role in promoting mammary tumorigenesis by influencing genomic stability at the centrosome level as well as reducing both basal and DNA damage-induced apoptosis.

MATERIALS AND METHODS

Cells

The p53-null mouse mammary epithelial cell (MEC) line was generated in the laboratory of Dr. Daniel Medina (Baylor College of Medicine), as described in Kittrell et al. (22). This line was derived from p53-knockout mice generated by Dr. Larry Donehower (Baylor College of Medicine) that were of mixed (129/Sv × C57Bl/6) genetic background (23). Cells were maintained in DMEM:F12 (Life Technologies, Inc., Grand Island, N.Y.) containing 2% ABS, 5 μg/ml gentamicin, 10 μg/ml insulin, and 5 ng/ml EGF. Stable cell lines were selected using this media plus 650 μg/ml G418. All cells were used at the lowest possible passage numbers, typically 7–11 in transient transfection experiments and 12–16 in stable cell experiments.

Plasmids

Transient transfections

The plasmid pBL120 contains a CMV-p53 172R-H minigene construct in pBluescript SK (Stratagene, San Diego, Calif.), while the plasmid pBL106B contains the wild-type p53 construct.

Stable transfections

The retroviral plasmid pL53NRNL (24), containing p53 172R-H under the control of the Mo-MuLV LTR and a *neo* selectable marker, was used in the generation of stable cell lines. Control stables were generated using a similar plasmid lacking the p53 construct.

Transient adenoviral transfection

Cells were transiently transfected using a replication-deficient adenovirus-mediated system. The adenovirus (25) was obtained from Dr. Nancy Weigel (Baylor College of Medicine). Cells were at 50–80% confluency when infected. At the time of infection, media were removed from the plates (10 cm dishes were used in these experiments) and replaced with 2 ml of serum-free media. Adenovirus–DNA complexes were prepared by incubating adenovirus with plasmid DNA for 30 min at room temperature in the dark, followed by a 30 min incubation (as above) with polylysine at a molar concentration equivalent to 125-fold the molar plasmid DNA concentration. Adenovirus/DNA/lysine complexes were immediately added to the serum-free media on the target cells and allowed to incubate for 2 h at 37°C. The viral solution was then removed and replaced with a normal volume of growth medium. Cells were harvested for analysis at the indicated number of days after transfection. Dishes (10 cm) were transfected with a total of 1 μg of DNA each, which included *bax* reporter plasmid (obtained from Dr. Moshe Oren, Weizmann Institute, Israel) or p53 construct and internal control plasmids where appropriate.

Stable transfection

293T retroviral packaging cells were transfected with pCL-Eco (26) and the appropriate retroviral plasmid using FuGene (Boehringer Mannheim, Mannheim, Germany). After 2 days, the virus-containing media were harvested and filtered (0.45 μm filter). Polybrene (Sigma, St. Louis, Mo.) was added to a final concentration of 5 mg/ml and the media were placed on target cells. The plates of cells were spun in a clinical centrifuge for 3 × 10 min at 1800 RPM, rotating the plates 60° after each spin to ensure complete coverage of the target cell plate. The medium was then removed and replaced with normal growth medium. Two days after infection, the medium was replaced with selective medium.

Western blotting

Western blots were performed in accordance with standard protocols (27). Membranes were blocked in 5% nonfat dry milk (NFDM)/TBST [Blotto] for 1 h, then incubated in

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either a 1:750 dilution of anti-p53 antibody (Santa Cruz, Santa Cruz, Calif.; sc-6243) or a 1:200 dilution of anti-p73 antibody (Novus Biologicals, NB 200-102) for 1 h at room temperature. After washing, the membrane was incubated in a 1:2000 dilution (3% NFDM) of anti-rabbit (or anti-sheep, respectively) biotinylated secondary antibody, followed by incubation in a 1:2500 dilution (3% NFDM) of horseradish peroxidase/streptavidin solution, treatment with ECL reagents, and exposure to film. The pan-ERK mAb used to normalize for protein content was obtained from Transduction Laboratories (Lexington, Ky.).

Immunocytochemistry

Cells to be immunostained were grown on lysine-coated circular coverslips and fixed in methanol at −20°C for 10 min, then washed and incubated in a solution of Blotto containing 1:50 polyclonal anti-p53 antibody (Santa Cruz, catalog # sc-6243) and 1:400 monoclonal anti-gamma-tubulin antibody (Sigma, catalog # T6557) for 3 h at 37°C. After washing with Blotto, they were further incubated with 1:500 Texas red-conjugated anti-rabbit secondary antibody and 1:200 FITC-conjugated anti-mouse secondary antibody (both from Molecular Probes, Eugene, Oreg.) in Blotto in the dark at 37°C for 1 h. The coverslips were then washed in TBST and mounted in DAPI solution (Vector Labs, Burlingame, Calif.; catalog # H-1200). Cells for p53/spindle double immunostaining were permeabilized in 0.5% Triton-X in PEM/PEG (80 mM Pipes, 1 mM EGTA, 1 mM $MgCl_2$, (pH 6.9) + 4% [8000 MW] PEG) and then fixed in 3.7% formaldehyde (all solutions in PEM/PEG) at room temperature for 15 min. After washing in PEM/PEG, coverslips were blocked in 5% NGS and incubated in 1:15 (in 5% NGS) TU27B mouse monoclonal antibody (gift of Dr. B. R. Brinkley, Baylor College of Medicine) and 1:50 p53 antibody as above for 1 h at 37°C. After washing in PEM/PEG, the cells were incubated in 1:100 (in 5% NGS) FITC-conjugated goat anti-mouse secondary antibody and 1:500 Texas red-conjugated goat anti-rabbit secondary antibody for 1 h at 37°C, washed, and mounted as above.

Centrosome analysis

Images from stained coverslips were captured with a Sony 3CCD color video camera attached to a BX-50 Olympus microscope with Adobe Photoshop software. All images were viewed using a 100× oil immersion lens coupled with a 10× objective. The number of centrosomes in cells with red nuclei (i.e., in cells expressing the transfected p53) was observed and recorded. Centrosome numbers in apoptotic cells were difficult to determine and therefore were not included in the analysis. The number of centrosomes in nonpositive cells from the same coverslips was recorded as an internal negative control. At least 1500 cells for each transfected population were counted at various cell passage numbers to ensure no passage number effect on centrosome results. Statistical analysis (by *t* test) was performed using the JMP Statistical Visualization software from SAS Institute, Inc (Cary, N.C.)

DNA damage protocols

Mitomycin C (MMC; Sigma) was applied to cells in media for the indicated period of time at the indicated concentrations. UV irradiation of cells was accomplished using a Stratalinker (Stratagene). A Gammacell 1000 Irradiator was used for exposure of cells to ionizing radiation. Doses of DNA-damaging agents and exposure times were chosen in accordance with accepted ranges in the literature (see, for example, ref 28).

Caspase assays

Cells were seeded into 96-well dishes at ~20,000 cells per well and treated as appropriate for each experiment. Each experimental point represents the average of at least four wells. At the time of assay, the media were aspirated and 30 μl of lysis buffer (10 mM Tris-Cl pH 7.5, 10 mM NaH_2P04/$NaHPO4$ pH 7.5, 130 mM NaCl, 1% Triton-X 100, 10 mM NaPPi) was added to each well. The plate was then incubated for 30 min on ice. After the incubation, a mixture of 3.6 μl substrate (Ac-DEVD-AMC caspase-3 fluorogenic substrate (PharMingen, San Diego, Calif.) and 216 μl of PAB (20 mM HEPES pH 7.5, 10% glycerol, 2 mM DTT) was added to each well and the plate was incubated in darkness at 37°C for 3–6 h. The quantity of AMC liberated after cleavage of the substrate by activated caspase-3 was measured using a spectrofluorometer (excitation wavelength 380, emission wavelength 430–460 nM). The protein content of each well was determined after the assay, and the plate readout was normalized accordingly to account for cell number variability and time of final incubation. The results are presented as the relative caspase activity/μg protein/h.

Colony assays

After irradiation, 500 cells were plated on each 10 cm dish and allowed to form colonies. After 10–14 days, colonies were stained with Coomassie blue; colony numbers ('colony' defined as >50 cells) were counted and statistical analysis was performed as above.

Luciferase assays

Cell extracts were made in 1× CAT assay lysis buffer (Boehringer Mannheim). Luciferase assays were performed as per manufacturer's instructions (PharMingen) using a Monolight 2010 luminometer (Analytical Luminescence Laboratory). Internal control (β-gal activity) assays were performed in 96-well plates. Twenty microliters of extract/well was incubated at 37°C in a solution containing magnesium (0.1M $MgCl_2$, 4.5M β-ME, 1.5 μl/well), 0.1M $NaHPO_4$ pH 7.5 (95.5 μl/well), and ONPG (4 mg/ml in 0.1 M $NaHPO_4$ pH 7.5, 33 μl/well) until a faint yellow color appeared (10–60 min). Plates were read at 420 nm on a spectrophotometer and units β-gal activity/μl protein were calculated.

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Chromosome staining

Cells were resuspended in 1 ml of media, to which 10 ml of 0.8% sodium citrate solution was added. The cells were then incubated at room temperature for 20 min, centrifuged, and resuspended in 1 ml of sodium citrate solution. Five milliliters of fresh 3:1 methanol:acetic acid solution (fixative) was added dropwise while gently vortexing. The cells were centrifuged and resuspended in 5 ml of fixative. Cells were dropped from a height of 4 inches onto slides slanted at a 30° angle and immediately followed with a few drops of fixative. After drying, cells were stained with a 1:1000 dilution of DAPI mount solution/phosphate-buffered saline for 1–2 min, rinsed, and mounted using solution lacking DAPI (Vector Labs, catalog # H-1000). Chromosomes were imaged as described above. At least 50 metaphase spreads were counted per cell genotype.

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SCE analysis

Confluent cells were split 1:5. Twenty-four hours after passage, 3 μg/ml BrdU was added to the medium and the cells were allowed to grow for an additional 48 h. The cells were refed with fresh regular medium for 3 h, then treated with 10 μg/ml demecolcine for 15 min to stop cell growth. Cells were washed, trypsinized, and centrifuged. The cell pellets were resuspended, and 10 ml prewarmed (37°C) 0.075M KCl solution was added dropwise while vortexing gently. One milliliter of fixative (see above) was then added and the cells were centrifuged. After aspiration of the media, the cells were resuspended in 10 ml of fixative and kept at room temperature for 30 min. The fixative was changed several times, and the cells were finally resuspended in ~0.5 ml of fixative. Cells were dropped onto slides as above and dried. Slides were then incubated in 0.1 mg/ml acridine orange (Sigma) solution for 5 min and rinsed well. The slides were mounted in 2.8% sodium phosphate anhydrous dibasic solution, pH 11.0, and visualized using a green (FITC) filter. For analysis, the number of breaks and the number of chromosomes were counted for each of 50 metaphase spreads per genotype, and the average break-per-chromosome ratio for each genotype was calculated.

RESULTS

Transient expression of the p53 172R-H mutant results in increased centrosome numbers

Loss of p53 expression in murine fibroblasts (29) and expression of the 172R-H p53 mutant in murine skin (13) result in aberrantly large numbers of centrosomes and aneuploidy. As aneuploidy is characteristic of mammary tumors arising in the WAP-p53 172R-H mice (12, 18, 20, 21), studies were undertaken to examine the effect of expression of this mutant p53 on centrosome number *in vitro*.

p53-null mouse MECs were transiently transfected with constructs encoding either wild-type p53 or the p53 172R-H mutant. Lipid-mediated transfection methods worked poorly with these cells, so an efficient adenovirus-mediated method (see Materials and Methods) was used. This resulted in a 20–40% transfection efficiency. Expression of wild-type or mutant p53 in transfected cells was initially verified by both immunocytochemistry and Western blotting (data not shown), and p53 expression could be detected in these cells for up to 5 days.

To determine the effect of transfected p53 on centrosome number in these cells with time, cells were transfected and populations grown on coverslips were fixed for analysis 2 h after transfection (day 0) and each day for the next 5 days (days 1–5). Fixed cells were then simultaneously immunostained for p53 and the centrosome component gamma-tubulin (see Materials and Methods). The double immunocytochemistry procedure permitted the identification of transfected cells for the analysis of centrosome number. The polyclonal p53 antibody used (see Materials and Methods) recognizes both wild-type and mutant forms of p53, and was therefore used for the analysis of both experimental groups.

Representative examples of centrosome and p53 staining in p53-null MECs transfected with either wild-type (**Fig. 1A**) or mutant (Fig. 1*B*) p53 are shown in Fig. 1. Statistical analysis of centrosome numbers in cells transfected with wild-type (Fig. 1*C*) or mutant (Fig. 1*D*) p53 as a function of the day after transfection was performed as described in Materials and Methods, and revealed that there were significantly more cells with supernumerary centrosomes in the population transfected with mutant p53 by day 3. (Fig. 1, compare the day 3–5 bars for the '2+'

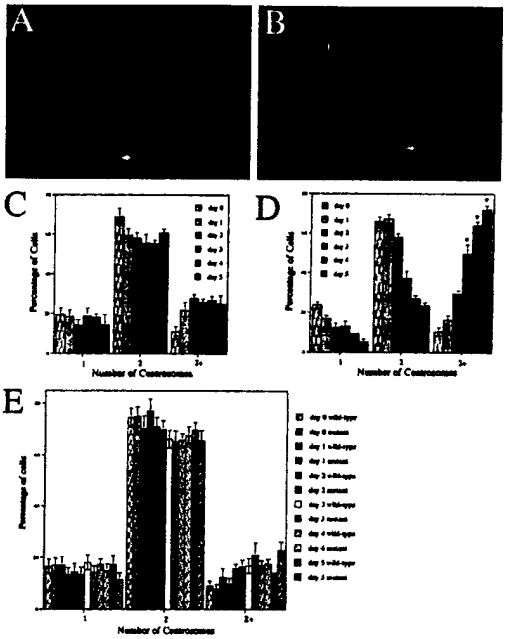

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Figure 1. Transient expression of the p53 172R-H mutant results in increased centrosome numbers. *A)* Representative nucleus positive for wild-type p53 (red) and associated with two centrosomes (green) 5 days after transfection. *B)* Representative nucleus positive for mutant p53 and associated with six centrosomes at the same time point. *C, D)* Statistical analysis of centrosome numbers in cells transfected with either wild-type p53 or the 172R-H mutant at time points ranging from the day of transfection (day 0) to 5 days after transfection (day 5). Asterisks indicate that in each case there are significantly ($P<0.001$) more cells with supernumerary (i.e., 2+) centrosomes in the mutant p53-transfected population than in the corresponding wild-type p53-transfected population at the same time point. *E)* Statistical analysis of centrosome numbers in untransfected cells from the same coverslips (both mutant p53- and wild-type p53-transfected coverslips). Error bars in all cases represent the standard error of the mean.

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category between panels *C* and *D*). The percentage of mutant p53-transfected cells with aberrant centrosome numbers increased each day after transfection, reaching a maximum of over 70% of transfected cells by day 5. A concomitant decrease in the numbers of cells with one or two centrosomes was observed. A small percentage of cells above background contain more than two centrosomes in the wild-type p53 transfectants (normal background levels are defined as ≤10% of cells; B. R. Brinkley, personal communication). However, the percentage of these wild-type p53-transfected cells with abnormal centrosome numbers plateaued at 20–25% by day 2 and remained relatively constant through day 5.

Centrosome numbers for nontransfected (p53-null) cells on the same coverslips at the same time points were also assessed and analyzed as an internal control (Fig. 1*E*). Nontransfected cells from wild-type and mutant p53-transfected plates exhibited very similar centrosome number profiles: ~20% with one centrosome, ~60% with two centrosomes, and ~20% with more than two centrosomes across all time points. This did not appear to vary significantly with cell passage number. These results essentially mirror those for wild-type p53 expressing cells depicted in Fig. 1*C* and indicate that the introduction of the gain-of-function p53 mutant into p53 null MECs results in centrosome abnormalities. Attempts were made to directly examine the effect of transfected wild-type or mutant p53 on cell ploidy by flow cytometry, but it was difficult to distinguish between transfected and nontransfected cells by this method. However, overall cell cycle profiles of populations transfected with mutant p53 and wild-type p53 were similar over time, even at time points at which centrosome number differences in the transfected populations were significantly different (data not shown).

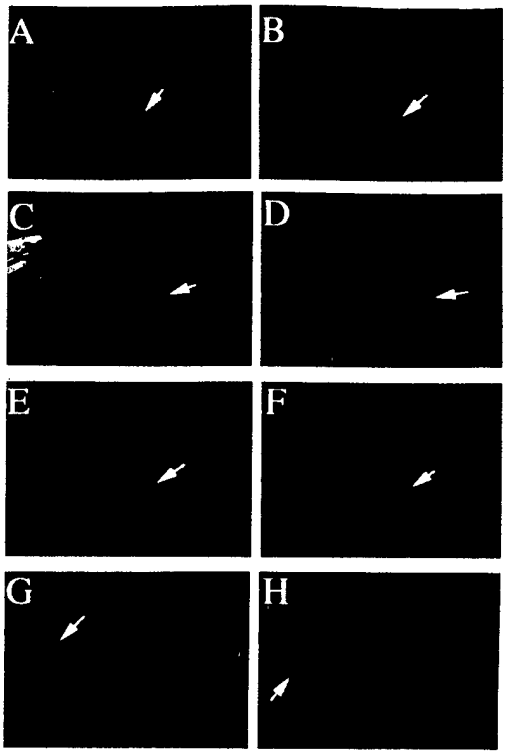

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Figure 2. Functional supernumerary centrosomes in cells transfected with the p53 172R-H mutant. *A, C, E)* Aberrant mitotic spindles found in cells transiently overexpressing p53 172R-H. *B, D, F)* Aberrant segregation of the cellular DNA in these same aberrant mitoses. By comparison, panels *G* and *H* depict a normal mitosis in an untransfected (i.e., p53 null) cell.

Functional supernumerary centrosomes in cells transfected with the p53 172R-H mutant

Although it is possible for cells with more than two centrosomes to form a bipolar spindle (30; K. Murphy, unpublished observations), one predicted consequence of the multiple centrosomes seen with this p53 mutant is the formation of multipolar spindles leading to aberrant genome segregation at mitosis. F2 As illustrated in **Fig. 2**, a small percentage of cells containing multipolar spindles was observed in these experiments. Double immunocytochemistry experiments were performed with simultaneous staining for p53 and α-tubulin, a component of the mitotic spindle. These experiments demonstrated that cells transfected with mutant p53 often contained multipolar spindles by the fifth day after transfection (Fig. 2*A/B, C/D,* and *E/F*) whereas only a small percentage of untransfected control (i.e., p53-null) cells had abnormal spindles at the same time point (Fig. 2*G/H*).

Moderately abnormal centrosome numbers in cells stably expressing p53 172R-H

To determine whether the abnormal centrosome numbers induced by expression of the p53 172R-H mutant persisted for longer than 5 days, cell lines stably expressing p53 172R-H or a control plasmid lacking p53 were generated as described in Materials and Methods, and their p53 status was confirmed by Western blotting (data not shown). No attempt was made to generate cells stably expressing wild-type p53, as stable overexpression of wild-type p53 leads to growth repression and/or apoptosis.

Mutant p53 was expressed at high levels in the stable cells (**Fig. 3***A*) whereas, as expected, the p53- F3

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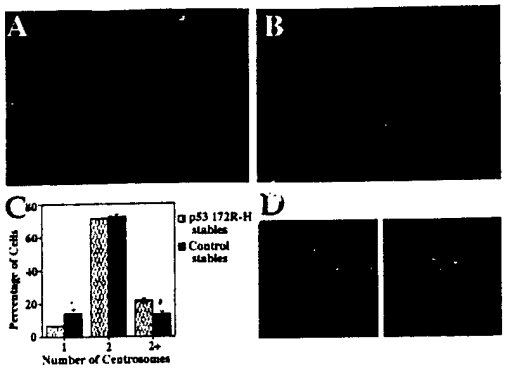

Figure 3. Moderately abnormal centrosome numbers in cells stably expressing p53 172R-H. p53 (red) and centrosome (green) staining is illustrated in p53 172R-H stable (*A*) and control (i.e., p53 null) stable (*B*) cells. *C)* Statistical analysis of centrosome numbers in p53 mutant stable and control stable cell lines. There were significantly more control stable cells with one centrosome (*$P<0.05$), and significantly more p53 172R-H stable cells with more than two centrosomes (#$P<0.02$). *D)* Two examples of p53 172R-H stable cells with supernumerary functional centrosomes (tripolar and quadripolar mitotic spindles, respectively).

null controls (Fig. 3*B*) displayed no detectable staining. Analysis of centrosome number in the mutant p53 stably transfected cells and controls (Fig. 3*C*) indicated that there were significantly more control cells containing one centrosome, and p53 172R-H cells with more than two centrosomes. Multipolar spindles were also observed to coexist with multiple centrosomes in some of the mutant p53 stable cells (Fig. 3*D*). However, based on the results from the transient transfection experiments, these differences in centrosome number were unexpectedly small. This raised the issue of the fate of the cells having multiple centrosomes and multipolar spindles after transient transfection with p53 172R-H.

As there were only moderately elevated numbers of stably transfected cells expressing the p53 mutant containing supernumerary centrosomes, it was hypothesized that many of the cells with multiple centrosomes nucleating multipolar spindles were undergoing apoptosis (30). To test this hypothesis, transient transfections were used to measure the level of activity of an integral apoptotic caspase, caspase-3. Some apoptosis was induced by the transfection process, but levels were equivalent between the cell types at day 1. Apoptosis was significantly higher ($P<0.001$) in cells transfected with mutant p53 than in mock-transfected cells by day 5 post-transfection (**Fig. 4**), and the difference approached statistical significance ($P<0.067$) by day 4. Caspase activity did not change significantly between day 1 and day 5 in the mock-transfected cells. These results were independently confirmed through use of a fluorescent TUNEL assay combined with p53 immunostaining, which indicated that p53 staining frequently colocalized with TUNEL staining in day 4 and day 5 mutant p53-transfected cells (data not shown). As this coincides with the time points in which peak numbers of multicentrosomal mutant p53-positive cells were observed, these results suggest that the majority of these multicentrosomal cells were undergoing apoptosis, accounting for the observation of relatively few multicentrosomal cells in stably transfected populations.

F4

Lower basal levels of apoptosis and increased resistance to DNA damage in p53 172R-H-expressing stable cell lines

Wild-type p53 is believed to play a role in DNA repair processes (31, 32) and p53 loss or mutation is known to have deleterious effects on these processes (33–35), although p53-deficient cells do not exhibit an increased mutation frequency after exposure to DNA-damaging agents (36). To test whether DNA repair was influenced by the presence of the p53 172R-H mutant in the stably transfected p53-null

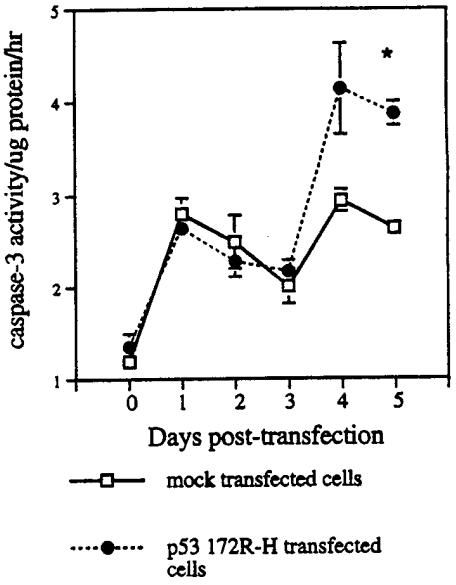

Figure 4. Cells transiently expressing p53 172R-H show substantially more apoptosis than mock-transfected cells by days 4 and 5 post-transfection. Caspase-3 assays were performed to measure apoptosis in MECs transiently transfected with p53 172R-H or control MECs on the day of transfection and each day thereafter until day 5. Numbers shown represent the average of at least four individual data points. The difference between the two cell lines is statistically significant (*$P<0.001$) by day 5.

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MECs, cells were treated with DNA-damaging agents and cell viability after treatment was ascertained by caspase-3 assays and/or colony assays as described in Materials and Methods.

Cells were treated with varying doses of mitomycin C (a DNA cross-linking agent), UV (which induces formation of cyclobutane dimers), or ionizing radiation (which induces double-strand DNA breaks) and cell survival was assessed at different subsequent time points. As shown in **Fig. 5**, treatment with all three of these DNA-damaging regimens yielded similar, if unexpected, results. Control cells treated with increasing concentrations of MMC contained significantly more caspase-3 activity (Fig. 5*A*) than correspondingly treated cells stably expressing the p53 172R-H mutant, indicating there was more apoptosis in control cells after MMC treatment. Treatment with UV radiation (Fig. 5*B*) appeared to exert a similar effect, especially at higher (30 or 50 J/cm^2) doses. Control cells treated with ionizing radiation (IR) also appear to die preferentially at all doses tested (Fig. 5*C*). The differences shown were statistically significant (P=0.05) for all three agents and at all concentrations/doses tested, including basal levels. Colony survival assays were also performed for the latter experiment. Control or p53 172R-H-expressing stable cells were exposed to either 5 or 8 Gy

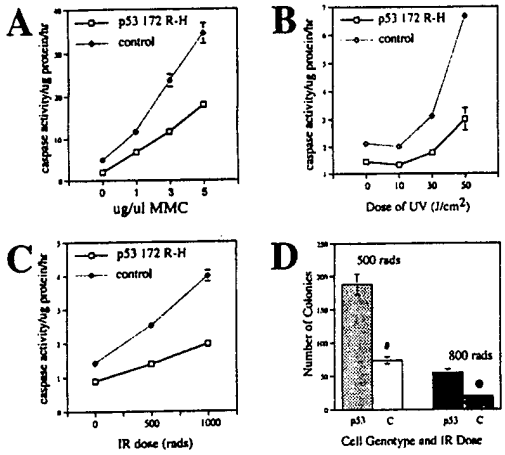

Figure 5. p53 172R-H-expressing stable cell lines have lower basal apoptosis and are more resistant to DNA damage induced by MMC (*A*), UV (*B*), and IR (*C, D*). *A–C)* The results of caspase-3 activity assays, representing time points of 2 days, 1 day, and 2 days after treatment, respectively. *D)* The results of colony assays, assessed 12 days after plating. Differences shown in panels *A–C* are statistically significant (P<0.05) at all doses and for all three agents. In many cases, error bars are small enough that they are not visible, although every point in each panel of this figure represents the average of at least four individual data points and has an error bar. Units are relative, therefore absolute caspase activities should not be compared from one treatment to another.

of ionizing radiation and allowed to form colonies as described in Materials and Methods. Colony counts indicated that the mutant-p53 expressing stables preferentially survived and formed more colonies at both doses tested (Fig. 5*D*). Figure 5*A–C* also shows that the p53 172R-H mutant can suppress basal apoptosis, as there is substantially more apoptosis in control than in mutant p53-expressing cells even without treatment with DNA damaging agents.

One potential explanation for these results is that other members of the p53 family are inducing apoptosis in the p53-null control cells, but that the mutant p53 protein exerts a dominant negative effect on that alternative proapoptotic pathway when stably expressed. It has previously been reported (37) that coexpression of the human p53 175R-H mutant inhibits the transcriptional activity of p73α on the *bax* promoter, thereby reducing the ability of exogenous p73α to promote apoptosis in p53-null H1299 cells. To examine the role of potential p73-mediated *trans*-activation of *bax* in apoptosis, a *bax*-luciferase reporter was transfected into cells stably expressing mutant p53 or control cells, which were left untreated or exposed to 30 J/cm^2 of UV irradiation to induce DNA damage and apoptosis. After 24 h, cells were harvested and luciferase activity was quantitated and normalized to an internal transfection control. These experiments indicated that the *bax* reporter is induced above basal levels by UV irradiation in both cell types and that there is significantly more reporter activity in both untreated and irradiated control cells than in their mutant p53-expressing counterparts (**Fig. 6*B***). Slightly elevated levels of p73 protein were observed in extracts of control cells relative to those in cells expressing mutant p53 in two separate experiments, although p73 levels did not appear to increase after irradiation (Fig. 6*A*). Endogenous p73 levels were extremely low in these p53-null MECs.

Increased chromosome number, but no change in sister chromatid exchange in cells stably expressing p53 172R-H

Mammary tumors arising in mice carrying the p53 172R-H transgene are frequently aneuploid (12, 18, 20, 21). To assess the effect of this p53 mutant on ploidy *in vitro*, metaphase spreads (**Fig. 7*A***) were prepared from p53 172R-H stably transfected or control cells as described in Materials and Methods, and chromosome numbers were analyzed. Both the control and p53 172R-H transfected cell lines averaged more than the normal 40 chromosomes per mouse cell. However, at equivalent passage numbers, there are significantly (P<0.05) more chromosomes (Fig. 7*B*) in cells stably transfected with the p53 172R-H mutant (mean=80, range=50–206) than in

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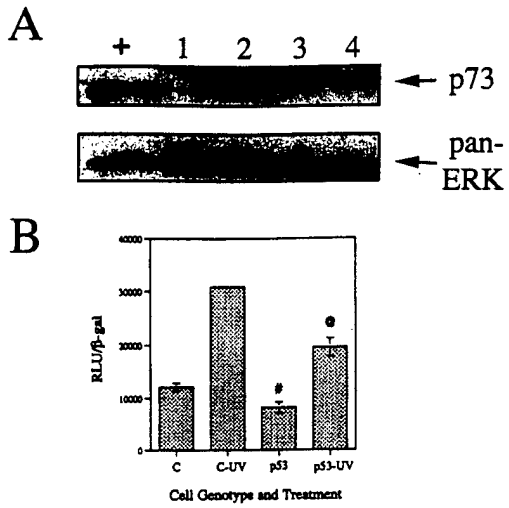

Figure 6. *A)* p53 172R-H-expressing stable cell lines appear to contain lower endogenous levels of p73 than control lines. A p73 Western blot with a COS cell positive control (+) followed by p53 172R-H-expressing stable cells, untreated and irradiated (lanes 1 and 2, respectively) and then untreated and irradiated control cells (lanes 3 and 4, respectively). Cells were exposed to 30 J/cm^2 of UV irradiation. The same blot was subsequently blotted with a pan-ERK antibody as a loading control, which showed no significant differences between lanes. *B) Bax* promoter activity is reduced in p53 172R-H-expressing stable cell lines. A *bax*-luciferase construct was transfected into mutant p53-transfected stable and control stable cells. Luciferase activity was quantitated in both cell types in the presence and absence of UV-induced (30 J/cm^2) DNA damage. There is significantly more bax promoter activity in both untreated and irradiated control (C) cells relative to their mutant p53-expressing counterparts ($P<0.017$ [#] and $P<0.001$ [@], respectively). The error bar in the lane labeled C-UV is too small to be visible.

control (i.e., p53-null) cells (mean=66, range=37–142).

One mechanism thought to promote abnormalities in DNA ploidy is uncontrolled sister chromatid exchange (SCE), which is influenced by p53 loss or mutation (38–40). Specifically, it has been suggested that the p53 172R-H mutant protein may improperly promote illegitimate homologous recombination (41, 42). Accordingly, the incidence of SCE (Fig. 7*C*) in p53 172R-H and control cell lines was assessed as described in Materials and Methods. Surprisingly, no significant difference in the average number of SCEs per chromosome was detected between the two stable cell lines (Fig. 7*D*). Average frequencies of 0.270 ± 0.015 and 0.292 ± .020 SCE/chromosome were observed for the p53 172R-H stably transfected and control cells, respectively. Of course, this does not preclude early changes in SCE or ~~that other~~ compensatory mutations ~~might not have occurred by~~ the time of analysis of these stably transfected cells.

DISCUSSION

Previous studies (12, 18, 20, 21) have shown that expression of the p53 172R-H mutant predisposes the mouse mammary gland to the development of aneuploid mammary tumors, but these studies did not address the specific mechanism(s) by which this occurs. The present studies performed in both transiently and stably transfected p53-null MECs suggest that this p53 mutant plays a dual role in mammary tumorigenesis, both negatively influencing apoptotic pathways and promoting the development of aneuploid cells.

Reduction in both basal and DNA damage-induced apoptosis

It was hypothesized that p53-null MECs stably overexpressing the p53 172R-H mutant would be defective in DNA repair and therefore more sensitive to DNA-damaging agents. However, these cells were in fact more resistant to DNA damage induced by MMC, UV, or IR over a range of doses. This suggests that, at least *in vitro*, this p53 mutant may promote cell survival after DNA damage. This p53 mutant also appeared to suppress basal apoptosis in the absence of DNA damage (Fig. 5*A–C*). It has been argued that p53 status does not significantly affect cellular sensitivity to DNA-damaging agents, in contrast to what is

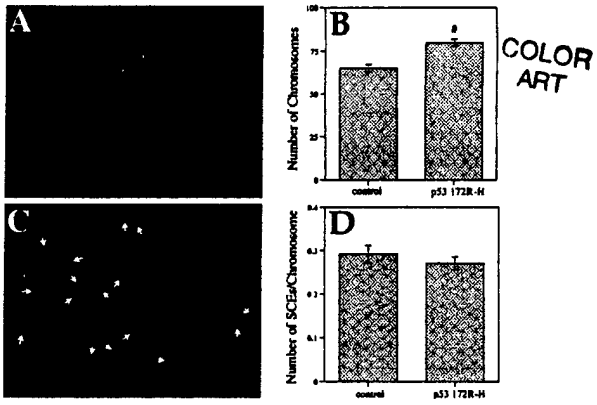

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Figure 7. Increased chromosome number, but no change in sister chromatid exchange in cells stably expressing p53 172R-H. *A)* Representative DAPI-stained metaphase spread from a cell stably expressing p53 172R-H. The statistical analysis of chromosome numbers in p53 172R-H stable cells vs. controls is presented (*B*) and indicates there are significantly more chromosomes in cells stably expressing mutant p53 (#, $P<0.001$). *C)* Representative metaphase spread illustrating occurrence of SCEs in the stable cell lines. Analysis of SCE frequency (number of SCEs/chromosome) indicated that there was no significant difference between the p53 172R-H and control stable cells (*D*).

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seen in short-term DNA damage assays (43), but to date no studies have directly compared the effects of DNA-damaging agents on survival in p53-null cells vs. p53-null cells expressing this mutant p53 isoform. The short-term and 'gold standard' (43) colony survival assay results here agree, which suggests that p53 172R-H may indeed be suppressing apoptosis in this p53-null MEC system.

How the 172R-H p53 mutant might suppress apoptosis is not clear. This p53 mutant may exert a dominant-negative effect on p63 or p73, other proposed members of the p53 tumor suppressor family that can *trans*-activate proapoptotic p53 target genes such as *bax* (3). Coexpression of the human p53 175R-H mutant is known to reduce the transcriptional activity of p73α on the *bax* promoter, thereby affecting the ability of ectopically expressed p73α to promote apoptosis in p53-null H1299 cells (37). In accordance with these results, both basal and DNA damage-induced *trans*-activation of a *bax*-luciferase construct was significantly diminished in MECs stably expressing the p53 172R-H mutant relative to levels seen in control p53-null cells. This has not been directly proved to result from p73 inactivation by the p53 mutant, but given the concordance of these results with those of Di Como et al., it is likely that the p53 172R-H mutant in this system is reducing apoptosis, at least in part, by blocking the activity of endogenous cellular p73. An increase in cellular p73 levels was not detected after DNA damage induced by UV irradiation, in keeping with the results of Fang et al., who used mitomycin C, doxorubicin, and actinomycin D to induce DNA damage (44), but in contrast to those of Gong et al. (45), who induced DNA damage using cisplatin. This suggests that there is DNA-damaging agent and possibly cell type specificity to p73 stabilization after DNA damage.

Chromosomal consequences of p53 172R-H expression

Mammary tumors arising in mice carrying the p53 172R-H transgene are frequently aneuploid (12, 18, 20, 21), and exogenous expression of this p53 mutant in p53-null fibroblasts results in hyperdiploidy (46). In these experiments, both the control and p53 172R-H stable cell lines averaged more than the normal 40 chromosomes per cell, probably because of the relatively high passage numbers required to generate stable cell lines and the inherent tendency of p53-null cell lines to be genomically unstable. However, at equivalent passage numbers, there are significantly more chromosomes (80 vs. 66) in cells stably transfected with the p53 172R-H mutant than in control (p53-null) cells, suggesting that the p53 mutant does exhibit a gain-of-function effect on chromosome number in this system.

It is believed that induction of SCEs represents the interchange of DNA replication products at homologous loci, involving DNA breakage and reunion (47, 48). Functional wild-type p53 interacts directly with RAD51 and its bacterial homologue RecA, key factors involved with homologous chromosomal recombination, and inhibits their function when appropriate (38). Defect in p53-mediated control of homologous recombination caused by mutation (38) or loss (40) of p53 may result in inappropriate chromosomal rearrangements and genomic instability. Previous experiments (41, 42) suggested that stable expression of the human p53 175R-H mutant increases the incidence of both spontaneous and radiation-induced homologous recombination, although meiotic recombination is unaffected by p53 loss (49). This suggested that more SCE might occur in MECs stably expressing the p53 172R-H mutant than in the control p53-null MECs, but SCE levels in these cells were in fact equivalent. The most likely reason for the disparity between these results and those reported previously is the lack of wild-type p53 in the MECs; in the previous experiments, the mutant p53 was probably functioning as a dominant negative. The p53 172R-H mutant does not appear to promote SCE beyond levels resulting from the absence of wild-type p53. However, both the control and mutant p53 stably transfected MECs appear to exhibit a mildly elevated SCE frequency relative to what is seen in normal mouse cells [0.185 SCE/chromosome (50) vs. 0.270 and 0.292 SCE/chromosome in the p53 172R-H stably transfected and control cells, respectively]. By comparison, SCE frequency is 1.94 ± 0.07 SCE/chromosome in cells derived from $Xrcc1^{-/-}/p53^{-/-}$ mouse embryos (51).

Centrosome amplification in mouse MECs expressing p53 172R-H

Transient expression of the p53 172R-H mutant protein in p53-null mouse MECs resulted in progressively increasing numbers of centrosomes per cell, reaching a maximum of over 70% of transfected cells with supernumerary centrosomes by day 5 post-transfection. By contrast, in transient transfections using wild-type p53, the percentages of cells with supernumerary centrosomes plateaued at ~20–25% by day 2 post-transfection, and similar results were seen with the internal control untransfected cells from the same coverslips at the same time points. Any cell containing more than two centrosomes is by definition aberrant (14). Loss of p53 in mouse embryo fibroblasts is known to result in extensive centrosome amplification (31–55% of cells with multiple centrosomes) by passage 2 (29). The relatively low background percentages of control p53-null MECs with aberrant centrosome numbers even after

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8–12 passages highlight a significant difference between fibroblasts and MECs. There are also species-related differences within individual cell types (see ref 52).

A variety of centrosomal kinases (e.g., STK15/BTAK; ref 53), phosphatases and other proteins (for example, the cdk2/cyclin E complex; ref 54, 55) have been implicated in centrosome duplication (for a review, see ref 30). *STK15/BTAK* is amplified and overexpressed in ~12% of human breast cancers, and expression of this kinase leads to centrosome amplification, chromosomal instability, and transformation *in vitro* (53). Cyclin E is an important regulator of S-phase entry in the mammalian cell cycle and is often overexpressed or deregulated in tumors, leading to centrosome amplification and/or genomic instability (54, 55). Cyclin E has been localized to the centrosome (55), as have wild-type p53 (56) and *STK15/BTAK* (53). It may be that the p53 172R-H mutant deregulates centrosome amplification by somehow influencing the activity of either *STK15/BTAK* or cyclin E-cdk2 at the centrosome.

A moderate percentage of p53-null MECs stably expressing the p53 172R-H mutant also contained more than two centrosomes. Functional supernumerary centrosomes might be expected to nucleate multipolar spindles, resulting in aberrant genome segregation. As expected, this phenomenon was observed in both the transient (Fig. 2) and stable transfectants (Fig. 3).

Brinkley and Goepfert have proposed a model to account for the continuing presence of supernumerary centrosomes in established breast tumors (30). In this model, centrosome amplification is initiated at a very early stage in cell transformation, followed by clonal selection of viable tumor progenitor cells. Major mitotic spindle aberrations resulting from the presence of multiple centrosomes would therefore be characteristic of early stages of tumorigenesis, and would result in significant loss or gain of chromosomes. Most of the initial progeny cells would then be removed by apoptosis, leaving an occasional tumor progenitor cell (30). The results presented in this study are consistent with this model and demonstrate that transient expression of the p53 172R-H mutant results in dramatic centrosome amplification and mitotic spindle aberrations followed by apoptosis. Stable expression of this p53 mutant is characterized by a much smaller percentage of cells with supernumerary centrosomes. Fully transformed cells with supernumerary centrosomes may survive and assemble bipolar mitotic spindles (30; K. Murphy, unpublished observations) by an unknown mechanism. It appears that in some cases cells manage to 'cluster' multiple centrosomes into only two spindle poles (57, 58).

In summary, a novel p53-null mouse mammary epithelial cell model has been used in studies investigating the role of p53 in mammary tumorigenesis *in vitro*. The p53 172R-H mutant plays a dual role in promoting mammary tumorigenesis. Expression of this mutant leads to centrosome amplification, predominantly followed by multipolar mitotic division and apoptosis. On rare occasions, these cells that have sustained mutant p53-related genetic abnormalities survive, and then may nucleate a pretumorigenic population. The presence of this p53 mutant reduced both basal and DNA damage-induced apoptosis in stably transfected cell lines. The combination of these two pathways presents a scenario in which cells are both more likely to sustain genetic aberrations and more likely to survive the presence of these aberrations, thus providing a plausible explanation for the frequency with which this p53 mutant is found in human breast cancers.

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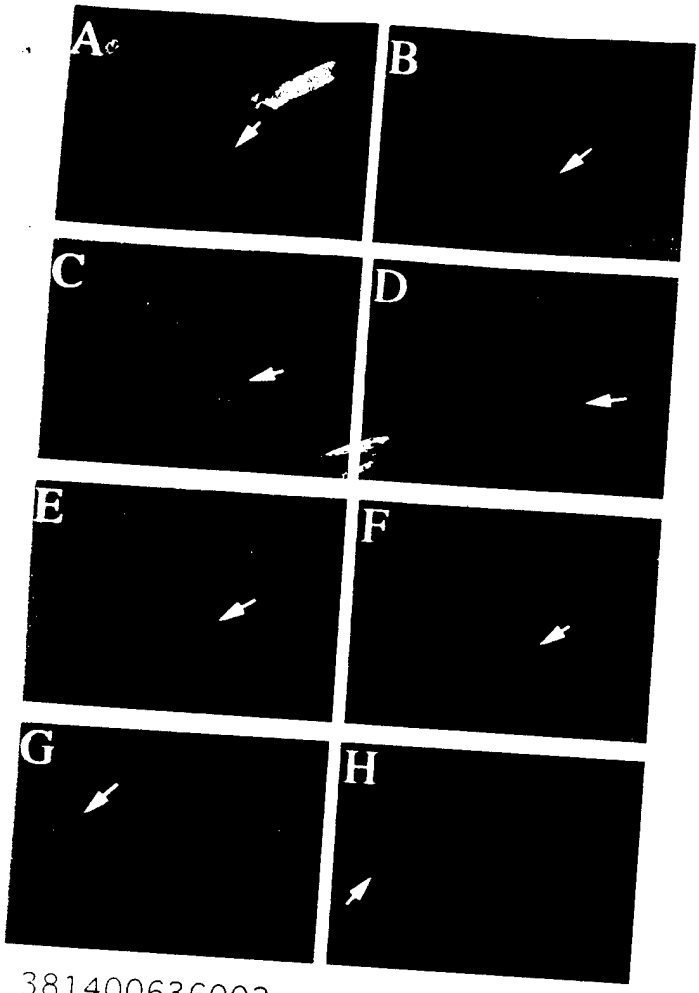

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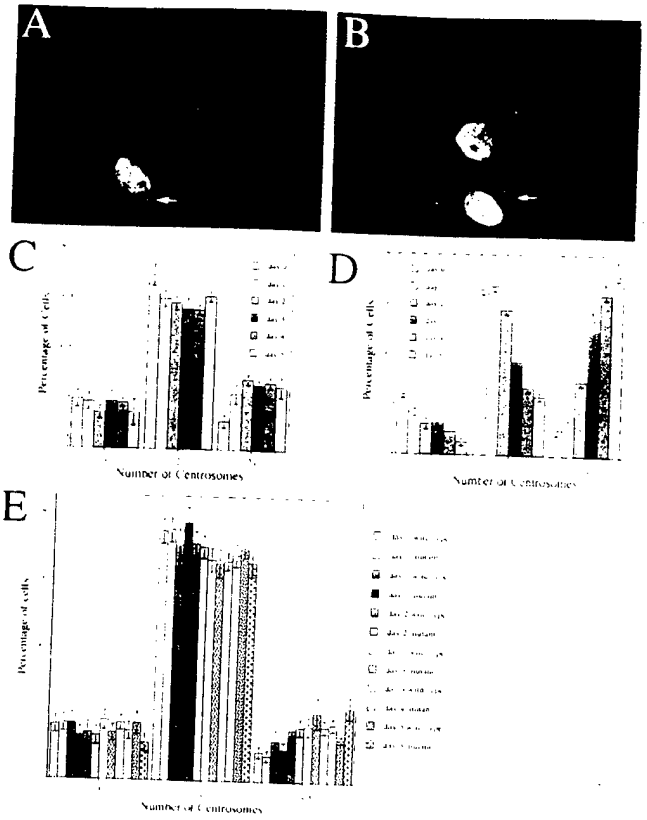

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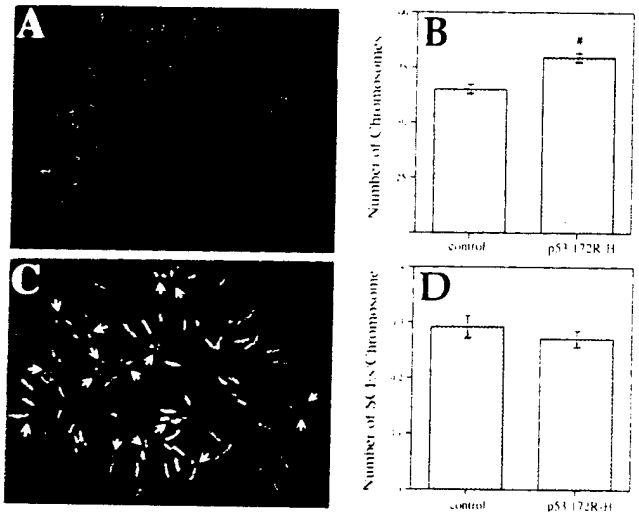

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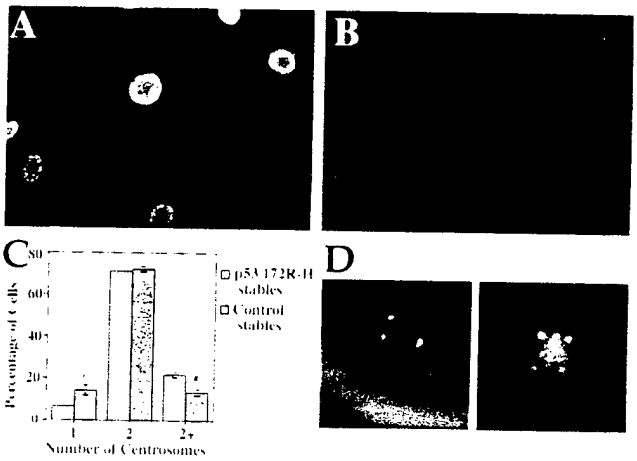

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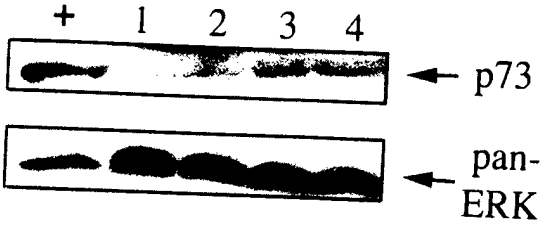
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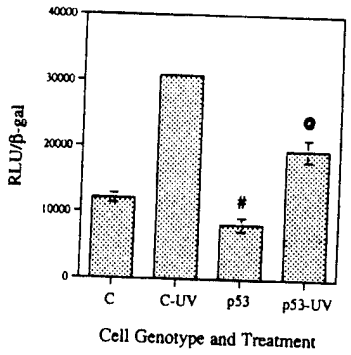
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